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FOR MAN
ANNUAL REPORT

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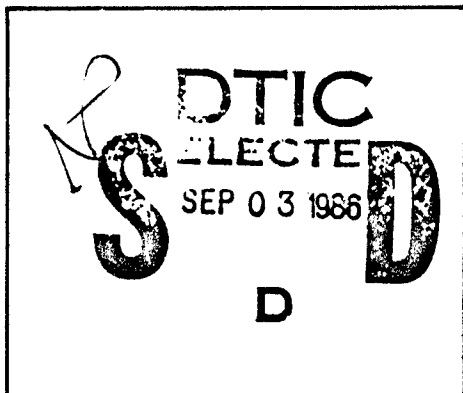
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In studies looking at the etiology of diarrhea among groups of U.S. adult students in Mexico we found that non-EPEC serotypes of enteroch adherent *E. coli* (EAEC) were isolated from 12% of diarrhea cases overall and from 30% of the patients from whom no other agents were identified. Six of 13 students from whom paired sera were collected showed a 4 fold or greater rise in antibodies in serum. Two strains of EAEC were selected for study in volunteers. In doses of 7×10^8 and 1×10^{10} viable cells, strain JM 221 produced a diarrheal illness in 4 of 8 subjects while 1 of 8 fed strain 189 developed a diarrheal illness. The test strains were recovered from stool throughout the study indicating intraluminal or intrainestinal replication of the test strains.

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Summary

Five Aeromonas hydrophila strains were selected for study from among more than 50 candidate strains collected from Thailand, Western Australia, Canada, and the United States. They were selected for the study because of possession of well characterized virulence properties or because they were clearly implicated in cases of human diarrheal illness. All were felt to be pathogenic by laboratory assays. The strains were tested biochemically and toxin production and hemagglutination patterns were characterized. Each strain was then fed to groups of 3 or 4 adult volunteers. The strains were shed by the volunteers only sporadically and except for two volunteers transiently passing unformed stools, a sustained illness did not develop. This study failed to show a relationship between virulence properties as we now understand them and pathogenicity of Aeromonas for human subjects.

In studies looking at the etiology of diarrhea among groups of U.S. adult students in Mexico we found that non-EPEC serotypes of enteroadherent E. coli (EAEC) were isolated from 12% of diarrhea cases overall and from 30% of the patients from whom no other agents were identified. Six of 13 students from whom paired sera were collected showed a 4 fold or greater rise in antibodies in serum. Two strains of EAEC were selected for study in volunteers. In doses of 7×10^8 and 1×10^{10} viable cells, strain JM 221 produced a diarrheal illness in 4 of 8 subjects while 1 of 8 fed strain 189 developed a diarrheal illness. The test strains were recovered from stool throughout the study indicating intraluminal or intraintestinal replication of the test strains.

Forward

For the protection of human subjects the investigator has adhered to policies of applicable Federal Law 45CFR46.

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Nature of Work Being Reported

The present report deals with 18 months of a total of 24 months concerning contract No. C-3024. The project has dealt with the establishment of the volunteer pool, the accumulation and evaluation of test strains and the administration of three doses of 5 A. hydrophila strains to volunteers and two dosage levels of two nonenteropathogenic serotype but enteroadherent E. coli strains.

Statement of the Problem

Approximately 50% of acute diarrhea is associated with the passage of unformed stools which are negative for all known enteric pathogens. Studies carried out in many regions of the world have shown that antimicrobial agents can be used to successfully treat or prevent a proportion of the illness offering indirect evidence that as yet undefined bacterial agents will prove to be important causes. Two leading bacterial candidates as potential causes of the undiagnosable illness are A. hydrophila and nonenteropathogenic but enteroadherent E. coli. A. hydrophila has recently been isolated in a high frequency of diarrhea cases which occur in Thailand, Western Australia, and Canada. The adherent E. coli have been isolated in 12% of cases of acute diarrhea cases occurring in U.S. adults studying in Mexico and may explain up to one third of the previously undiagnosable illness. The present study was designed to establish the virulence of these strains for man and to characterize the pathogenesis of infection for those capable of producing illness. Additionally, this volunteer program remains one of the few facilities in the country where enteric infections can be induced in man for the purpose of studying experimental infection. Priority for future study will be given for enteropathogens of interest to the U.S. Army.

Background

Diarrheal diseases are a major cause of morbidity in all areas of the world and mortality in developing regions. A large proportion of acute diarrhea is undiagnosable etiologically, yet these cases often respond promptly to antimicrobial therapy (1-3). This finding suggests that undefined bacterial agents may be responsible for acute diarrhea.

Various strains of Aeromonas hydrophila possess characteristics associated with virulence of enteropathogenic bacteria. Most strains produce a heat-labile cytotoxin as well as enterotoxins (4-6). A recent report indicates that a cholera-like toxin may be produced as well by Aeromonas strains (C Houston, ASM 1984). A. hydrophila strains are also capable of hemagglutinating human erythrocytes (7,8). With pathogenic E. coli this property is often associated with fimbriae that are able to bind to human epithelial cells.

A. hydrophila is currently considered a human pathogen and has been for some time (9,10). More recently, strains of A. hydrophila have been associated with cases of diarrhea in children and adults (4,5,11,12). However, the organism is ubiquitous and is frequently found in water and in stools of healthy persons (4,6,9,13,14). Although a statistical relationship exists between illness and fecal recovery of A. hydrophila, definite proof of pathogenicity for man is lacking. Oral administration of whole cultures to rhesus monkeys, generally felt to be an animal model closely resembling human infection, failed to cause diarrhea (4). This study was designed to determine if A. hydrophila strains were capable of causing diarrhea in humans when

challenged with whole, viable cultures.

We have conducted a number of studies in Mexico looking at the epidemiology, etiology, therapy and prevention of diarrhea in young adults from the U.S. during short term stay there (1,2,15,16). This setting closely resembles the situation of U.S. military populations during short term relocation in the developing world. We became interested in the observation that antimicrobial agents could successfully treat and prevent the diarrhea even when an agent was not identified in stool (1,2,17). Through a study of serotype and virulence properties of the E. coli isolated from diarrheal stools we identified an agent heretofore not described to be associated with diarrhea (18,19). Initially, we failed to identify common serogroups among the E. coli isolated from diarrheal stools, and furthermore found that enteropathogenic E. coli serogroups were encountered only rarely. Of perhaps greater interest, however, E. coli from illness cases commonly were shown to adhere to HEp-2 cells, a model of pathogenicity for EPEC strains (20). Strains of so called enteroadherent E. coli (EAEC) were found in 42 of 349 (12%) of illness specimens and 7 of 121 (6%) of controls. When looking at the group of students without other agents, 26 of 89 (29%) possessed an EAEC. Thirteen students with an EAEC in diarrheal stool furnished paired sera for serologic study. Six of 13 (46%) showed a four fold or greater rise in antibody to somatic antigen of the isolated E. coli strain (19). The present study was designed to confirm the virulence of EAEC for man and to determine the pathogenesis of infection. These studies offer evidence of a new cause of diarrhea and raise important questions as to the current thinking about relationship of serotype to pathogenicity among E. coli.

Approach to Problem

Aeromonas Studies

Test Strains

Five human isolates of A. hydrophila were selected for virulence assays and volunteer studies (table 1). All strains were lyophilized upon receipt and stored at room temperature. A new lyophile of the same lot was used for each study.

Biochemical Characterization

All strains were confirmed as A. hydrophila using the API-20E identification system (Analytab Products, Plainview, N.Y.). In addition, strains were plated on DNase test agar (Difco, Detroit, MI.) supplemented with oxgall (Difco) and crystal violet (Difco), as previously described (21).

Antimicrobial Susceptibility Testing

Antibiograms were determined using a standard agar diffusion (Kirby-Bauer) method. All strains were tested for susceptibility to ampicillin (AM-10), tetracycline (TE-30), trimethoprim/sulfamethoxazole (SXT), gentamicin (GM-10), furazolidone (FX-100), doxycycline (D-10), and sulfisoxazole (G-.25). Standard ATCC E. coli, S. aureus, S. fecalis and Ps. aeruginosa were used as control strains.

Toxin Assays

Hemolysin activity was assayed using sheep and rabbit blood agar plates (Remel Media, Houston, Texas). Strains were inoculated onto both blood agar plates. After an overnight incubation, the plates were examined for beta hemolysis.

Cytotoxin activity was measured in Y-1 adrenal cells (YAC). Cell free supernatants, prepared as previously described (21, 22) were added in 100 μ l amounts to confluent YAC monolayers. Following an 18-24 hour incubation, the monolayers were examined for cytotoxic activity (detached cells). Supernatants which caused 100% cytotoxicity were considered positive.

Enterotoxin activity was measured as previously described using the suckling mouse assay (21) and the rabbit ileal loop model (23). Cholera toxin cross-reactive factor (CTCRF) was measured in a ganglioside ELISA (22), using purified cholera toxin to produce a standard curve.

Invasiveness or the ability to invade tissues was assayed in the Sereny eye model (24). Overnight CYE broth cultures (0.1 ml) were inoculated into the eyes of adult Hartley strain guinea pigs (Charles River, Wilmington, MA). The animals were examined for keratoconjunctivitis for 8 days. A second method was used to screen for possible invasive capability by the test strains. Overnight CYE broth cultures (1.0 ml) were injected into ligated rabbit ileal loops. The animals were sacrificed after 18 hours and the ligated loops were examined for evidence of invasion and fluid secretion. In both assays, virulent S. sonnei 53GI was used as a positive control.

Hemagglutination Assays

Mannose-sensitive and mannose resistant hemagglutination patterns were determined using the method of Evans et al. (25). Human (type A), bovine (Flow Laboratories, McLean, Va), chicken (Flow), Monkey (Flow) and guinea pig (Flow) erythrocytes were tested in the presence and absence of D-mannose (Sigma Chemical Corp., St. Louis, MO).

Selection of Enteroadherent E. coli for Testing in Volunteers

Two strains of EAEC were selected for further study in volunteers, JM 221 and JM 189. They were further evaluated since: they did not produce LT or ST; did not produce keratoconjunctivitis in the guinea pig eye; did not belong to EPEC serogroups; were the sole pathogen isolated from cases of diarrhea; and they each were associated with a 4 fold or greater rise in serum antibody to the organism's somatic antigen during the episode of illness.

Volunteer Challenge Studies

Volunteers were identified through advertisement in local university newspapers and the Houston Chronicle (city-wide circulation). The advertisements instructed potential volunteers to call a phone number to learn more about the study from a recruiter. Volunteers were then asked to come to Methodist Hospital to hear details about the study a second time and to be scheduled for medical screening. During the screening process, a medical history form was completed and reviewed by a physician and a chest x-ray, EKG, and blood chemistry profile were performed. Consenting healthy adults were

admitted and confined to the Methodist General Clinical Research Center. A prechallenge admission stool sample was cultured for enteropathogens as described (21).

Volunteers abstained from eating and drinking for the 90 minutes prior to and following oral challenge with test organisms. In a double blind study, groups of 3 or 4 volunteers were given 2 grams of NaHCO_3 in 150 ml sterile distilled water as follows: 120 ml of the bicarbonate solution was swallowed and 5 minutes later, the remaining 30 ml of bicarbonate liquid plus the challenge inoculum at predetermined levels were rapidly swallowed. The volunteers were carefully monitored for signs of diarrhea defined as 3 or more unformed stools in 24 hours or 2 or more unformed stools in 24 hours with systemic or enteric symptoms. All stools passed were collected and cultured for A. hydrophila or EAEC.

Results

Aeromonas Studies

Biochemical Characterization and Antimicrobial Susceptibility

All 5 strains were confirmed as A. hydrophila biochemically. The occurrence of several biochemical properties was noted, as potential indicators of virulence (table 2). All of the strains gave a positive Vogues-Praskauer reaction; only two of the strains produced lysine decarboxylase; all were capable of hydrolyzing esculin and produced DNase. The strains were quite susceptible to antimicrobial agents used to treat acute bacterial diarrhea.

Toxin Production

All strains were hemolytic for sheep and rabbit erythrocytes and cytotoxic for YAC. Three strains (6V, B158, and 3647) produced fluid accumulation in the suckling mouse and rabbit ligated ileal loop assays. All five test strains produced a cholera-like toxin cross reactive factor in a ganglioside ELISA.

Hemagglutination Assays

The strains failed to show mannose-resistant hemagglutination with the erythrocytes obtained from five animal species. Strain B158 showed mannose-sensitive agglutination for guinea pig erythrocytes.

Invasiveness

None of the A. hydrophila strains were able to induce keratoconjunctivitis in guinea pigs. All 5 strains did, however, produce fluid secretion in the rabbit ileal loops with a purulent hemorrhagic discharge.

Volunteer Challenge Studies

Using the 5 Aeromonas strains, we failed to demonstrate the development of a diarrheal illness in 55 of 57 volunteers even with the doses of 10^{10} colony forming units for three of the strains (table 4). Three strains (B158, SSU and 3284) were not recovered from the stools of the volunteers. Strain 6V was recovered from 11 of the 20 volunteers challenged. One of the volunteers developed mild diarrhea (passage of 6 unformed stools over 12 hours associated

with a brief period of nausea, vomiting, anorexia and malaise) 48 hours after ingesting 3×10^9 colony forming units. He was not treated and failed to develop a progressive enteric illness. A small bowel biopsy obtained shortly after passing the unformed stool was normal histologically. No illness occurred among the 4 individuals ingesting a higher dose of 6Y (4×10^{10}) casting doubt on the importance of the mild illness in the one volunteer seen at the lower dose. Strain 3647, earlier identified in a diarrheal stool from a patient in Australia, was recovered from 3 of the 16 volunteers challenged. One volunteer receiving a dose of 10^7 cells passed 3 soft stools over an 18 hour period of time. He had mild abdominal cramps, but did not excrete the test organism. Illness did not occur as the dose was increased and the organism was administered to additional volunteers.

Table 5 summarizes the results obtained when volunteers were fed two dosage levels of the two EAEC strains. When the two strains were ingested in a dose of 7×10^3 cells by the 8 volunteers, 2 of 4 subjects experienced diarrhea as a result of exposure to strain 221 and 1 of 4 developed diarrhea following ingestion of strain 189. Three of 4 subjects receiving strain 189 in the lower dose experienced malaise, myalgias and abdominal pain. When the dose was increased to 10^{10} cells, 2 of the 4 individuals ingesting strain 221 experienced a diarrheal illness with an incubation period of 28 hours, and 3 of 4 experienced additional symptoms of enteric infection (malaise, vomiting, abdominal pain). Illness did not develop in the 4 volunteers receiving the higher dose of strain 189. Sixteen of 16 volunteers fed either strain at the two dosage levels excreted the test strain for 3 days or longer indicating replication of the strains in the gut.

Discussion and Conclusions

Aeromonas

A. hydrophila, an organism found commonly in the environment, has been statistically associated with diarrheal disease in man in a limited number of parts of the world. Its pathogenicity for selected patients is certain particularly in skin infections and septicemia. We tested 5 representative strains of A. hydrophila based on their possession of well characterized virulence properties as well as their association with diarrheal disease. Diarrheal illness failed to occur in 57 volunteers fed varying doses of the characterized strains. The strains did not efficiently colonize the gut of the volunteers as demonstrated by the resultant fecal shedding patterns.

One of the two following conclusions appear to explain the results of the present study:

1. Virulence properties of A. hydrophila as we now understand them - biochemical characteristics and production of hemolysin, cytotoxin, and enterotoxins are insufficient to explain virulence for man.
2. Widespread immunity to A. hydrophila exists among adults from Houston, Texas.

We feel that the latter is not a reasonable explanation for failure to produce illness in view of the rarity of isolating Aeromonas from diarrheal stools of infants and children from Houston studied by our group over the past 10 years (26,27). Perhaps we are at the point with Aeromonas, in terms of

understanding its virulence characteristics, where we found ourselves in the early 1970's for Escherichia coli. It is possible that the strains of Aeromonas we tested lack the necessary fimbriae to initiate colonization, the first step in pathogenesis. Even though the strains possessed hemagglutinins, which may be associated with fimbriae, these fimbriae may not be intestinal epithelial cells adhesins for Aeromonas. Previously recommended biochemical testing (6) was not useful in differentiating strains with virulence for man. Additional virulence properties of Aeromonas strains need to be sought and identified before future volunteer studies are likely to be rewarding. Also, we know that enteric bacteria normally pathogenic for only infants (ie, EPEC) can produce diarrhea in adults when given in the doses employed here (28).

Enteroadherent E. coli

Our studies have indicated that Escherichia coli which are identified only by their HEp-2 cell adherence property are as commonly associated with acute diarrhea in young adults traveling to Mexico as are strains of shigella. That these strains actually caused the illness in this setting is suggested by a variety of findings: 1. The agents often were the sole agent identified in diarrheal stools; 2. They were isolated in a lower frequency from asymptomatic individuals when compared to illness cases; 3. Humoral antibody development to somatic antigens of the organism commonly occurred during infection; and 4. A limited number of volunteers exposed in Houston to 10^8 - 10^{10} cells experienced clinical illness resembling the disease originally studied in Mexico.

Table 1. Source of Strains of
Aeromonas hydrophila

<u>Strain</u>	<u>Location</u>	<u>Site of Infection</u>
6Y	Bangkok (Echeverria)	Stool (asymptomatic)
B158	Perth (Gracey)	Wound
3647	Perth (Gracey)	Stool (diarrhea)
SSU	U.S.-C.D.C. (C. Houston)	Stool (diarrhea)
3284	Perth (Gracey)	Stool (diarrhea)

Table 2. Biochemical Characterization and Antimicrobial
Susceptibility of A. hydrophila Strains

Strain	Biochemical Reactions				Antibiogram*						
	Voges Praskauer Reaction	Lysine Decarb. Production	Esculin Hydrolysis	DNase Production	Am	Te	SXT	GM	Fx	DX	G
6Y	+	-	+	+	S	S	S	S	S	S	S
B158	+	-	+	+	R	S	S	S	S	S	S
3647	+	-	+	+	R	S	S	S	S	S	S
SSU	+	+	+	+	S	S	S	S	S	S	S
3284	+	+	+	+	R	S	S	S	S	S	S

*Am - Ampicillin, Te - Tetracycline, SXT - Trimethoprim/sulfamethoxazole, GM - Gentamicin, Fx - Furazolidone, DX - doxycycline, G - Sulfisoxazole.

Table 3. Toxins Produced and Hemagglutination Patterns
of A. hydrophila Strains

Strain	Toxin Production					Hemagglutination Pattern				
	Hemolysin	Cytotoxin	Suckling Mouse	Rabbit ileal loop	CTCRF	Red Blood Cells Tested				Guinea Pig
6Y	+	+	+	+	+	-	-	-	-	-
B158	+	+	+	+	+	-	-	-	-	MS
3647	+	+	+	+	+	-	-	-	-	-
SSU	+	+	-	-	+	-	-	-	-	-
3284	+	+	-	-	+	-	-	-	-	-

*Mannose Sensitive hemagglutination only.

Table 4. Administered Dose and Excretion of

A. hydrophila in Volunteers

Strain	Challenge Dose	Number Volunteers	Number Shedding Test Strain	Diarrhea ⁺
6Y	2x10 ⁴	4	1	0
	1x10 ⁶	4	1	0
	7x10 ⁷	4	4	0
	3x10 ⁹	4	3	1
	4x10 ¹⁰	4	2	0
B158	6x10 ⁴	4	0	0
	2x10 ⁷	4	0	0
3647	1x10 ⁷	4	0	1
	4x10 ⁷	4	0	0
	2x10 ⁹	4	2	0
	3x10 ¹⁰	4	1	0
SSU	4x10 ⁸	4	0	0
	5x10 ⁸	3	0	0
3284	3x10 ⁸	3	0	0
	1x10 ¹⁰	3	0	0

⁺ 3 unformed stools/24 hrs or 2 unformed stools/24 hrs with systemic or enteric symptoms.

Table 5. Challenge Dose, Strain Excretion and
Occurrence of Symptoms - Two Enteroadherent
E. coli Strains

Strain	Challenge Dose	Number Volunteers	Shedding Organisms (3 Days)	Enteric Symptoms*	Diarrhea+
JM 189	7×10^8	4	4	3	1
	6×10^{10}	4	4	0	0
JM 221	7×10^8	4	4	0	2
	1×10^{10}	4	4	3	2

*Fever, myalgias, malaise, abdominal pain or cramps.

+ 3 unformed stools/24 hr or 2 unformed stools/24 hrs with systemic or enteric symptoms.

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Figure 1. An adherent E. coli strain in the HEp-2 assay (right).
A nonadherent E. coli strain in the HEp-2 assay (left).